Enhancing Malaria Vaccine Development by the Naval Medical Research Center

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Phase II Progress Report

Milestone 1: Plasmid Impregnation and Characterization

1. Executive Summary

A biopolymeric delivery system provided an effective new method for the introduction of plasmid DNA vaccines designed against malaria. During Milestone 1 of this Phase II project, the feasibility of this vaccine/polymer system was ascertained via characterization of plasmid impregnation and particle morphology. Particles of poly(D,L-lactide-co-glycolide) (PLGA) with incorporated DNA plasmid were developed for systemic administration of DNA plasmids for use as a malaria vaccine. Objectives in Milestone 1 included impregnation of intact plasmid DNA within a PLGA excipient and reduction in size of the pDNA/polymer matrix followed by monitoring release of the plasmid from the matrix. The outcomes of Milestone 1 studies demonstrated that the incorporated plasmid was intact, that the plasmid is incorporated within the matrix, and that polymer particle sizes were capable of being engulfed by antigen presenting cells. The evaluation of the particle size distribution demonstrated that 90 percent of the particles were less than 20 μm and the median particle size was on the order of 8 μm. An *in vitro* release study evaluated incorporation of the plasmid and determined that the "burst effect" or immediate release of the plasmid upon exposure to an aqueous environment was controlled. The biopolymer system promoted sustained release of the plasmid for a period up to 14 days with plasmid release detectable through 49 days. The release profile of the plasmid was modeled to estimate an effective dose of the plasmid/PLGA system for future use in preclinical malaria models. The plasmid/PLGA system demonstrated potential efficacy *in vitro* and development continued to produce a new vaccine system against malaria.

2. Background and significance

2.1 Malaria Vaccine Development

Each year approximately 300 to 500 million people are infected with malaria and each year 1.5 to 2.7 million people die from this disease (Gardner et al., 1998; WHO, 1997). Since World War II, the struggle against malaria has gone through several stages. The first stage involved a massive effort aimed at eradicating the vector. The second stage was the development of antimalarial drugs based on quinine derivatives and alternatives (Shulemann, 1932; Corell et al., 1955). Due to introduced drug resistance, Miller and Hoffman (1998) stated that vaccination represents the best potential for control of the disease. The third stage of malaria control, then, recognizes the limitations of vector control and chemotherapy. In this regard, a current emphasis is on development of DNA-based vaccines against one or more of the developmental forms of the malaria parasite. Various strategies have been explored for implementing DNA-based vaccines.

Vaccines may prove beneficial to a wide range of populations. Proposed goals aim to prevent disease in foreign travelers and residents in low transmission areas such as India and reduce disease in high transmission areas

such as sub-Saharan Africa. Even vaccines demonstrated to provoke only low levels of antibodies might be useful in priming the immune system. Subsequent natural infection would help reduce the disease in high-risk populations such as children and pregnant women of Africa (Hoffman et al., 1998). The potential and applicability of malaria vaccines as a treatment method has led to the development of a number of candidates. Several additional candidate vaccines are expected in coming years upon sequencing of the *P. falciparum* genome (Gardner et al., 1998).

Vaccine trials have progressed from mice (Doolan et al., 1996) to monkeys (Wang et al., 1998) and into humans (Stoute et al., 1997; Wang et al., 1998). Malaria vaccines work by inducing the production of CD8+ T-cells that kill infected hepatocytes. Immunity stems from recognition of peptides present on the surface of infected hepatocytes by CD8+ T-cells that mediate infected cell elimination. Doolan et al., (1996) demonstrated partial protection ranging from 8 to 75 percent among various breeds of mice inoculated intramuscularly with DNA encoding for the *Plasmodium yoelii* circumsporozoite protein (PyCSP). Protection ranging from 80 to 90 percent was conferred onto mice by injection of a combination of plasmid vaccines namely PyCSP and *Plasmodium yoelli* hepatocyte erythrocyte protein 17 (PyHEP17). The success of the combination was attributed to a circumvention of genetic restrictions that lessened protective immunity mediated by CD8+ T-cells. Clinical vaccines are likely to include several protein-inducing plasmids to overcome genetic restrictions and handle parasite polymorphism.

Wang et al., (1998) applied the concept of multigene immunization in a study involving rhesus monkeys. Monkeys were injected three times at weeks 0, 4 and 16 at each of four intramuscular sites. The induction of antigen-specific antibodies required multiple immunizations. No antibodies were detected in any of the subjects following the first immunization. However, 8 of 12 monkeys demonstrated antibody production following the second immunization and 11 of 12 after the third immunization. Furthermore, 8 of 12 animals expressed CD8+ T-cell responses to all of the delivered epitopes and three additional animals showed CD8+ T-cell responses to all but one. These results help to support the effectiveness of the multiple epitope immunization approach.

Based on the encouraging results in nonhuman primates, Hoffman et al., (1997) proposed a plan to clinically test a multigene malaria vaccine in humans. Twenty malaria naïve volunteers were given three immunizations of the *P. falciparum* liver-stage DNA vaccine. The induction of CD8+ T-cell against the expressed protein was monitored by collection of peripheal blood mononuclear cells. Wang et al., (1998) reported that immune responses were detected in doses as small as µg, but doses ranging from 500 to 2500 µg elicited responses to approximately 70 percent of all of the peptides studied. In general, the magnitude of the immune response was also reported to be significantly higher than observed in humans exposed to conventional irradiated sporozoites or natural infection alone. Le et al., (2000) conducted safety studies and subjects observed mostly mild systems through one year following immunizations. However, the effectiveness of the vaccine was questioned, as there were no

detectable antigen-specific antibodies present despite an induction of CD8+ T-cell response. Stoute et al., (1997) conducted independent clinical trials of *P. falciparum* vaccines with mixed results. Human volunteers were vaccinated and then exposed to infection causing development of malaria in 100 percent of control subjects. Two vaccine formulations had little effect as the majority of volunteers contracted the disease, but a third formulation prevented malaria in seven of eight volunteers. Further studies were indicated to determine vaccine safety and reasons why the third formulation may have been more successful than others.

There have been measures to improve vaccine efficacy. Sedegah et al., (1998) demonstrated increases in protection by priming with the malaria vaccine and boosting with recombinant vaccinia. Malaria challenged mice demonstrated protection in 69 percent of the subjects boosted with vaccinia PyCSP, versus 44 percent of animals immunized with the DNA vaccine alone. Subsequent studies by Sedegah et al. (2000) combined both boosting with vaccinia PyCSP and coadministration of a plasmid expressing murine GM-CSF. Priming with PyCSP plasmid DNA and plasmid GM-CSF was demonstrated to confer protection to 100 percent of challenged mice dependent upon amount of recombinant vaccinia delivered during boosting.

2.2 Delivery of Plasmid-Based Vaccines

The use of homo- and copolymers of lactide and glycolide (PLGA) for biomedical applications is well-established and is based on the biocompatibility of these materials and their degradation products, lactic and glycolic acids (Visscher et al., 1985). Rates of degradation and release of incorporated active agents are dependent both on the molecular weight of the polymer and on the lactide-to-glycolide ratio. A sample list of proteins and peptides, which have been incorporated into PLGA has been compiled by Cleland and Langer (1994).

Traditional emulsion techniques for PLGA vaccines use blenders to generate the emulsions. However, the energy of this process results in some degradation of the DNA. As a consequence a large portion of the supercoiled material was degraded to the open circle or linear form. The damage is a consequence of the shear forces acting on the liquid components of the emulsion. The patented process proposed here does not involve emulsion formation and can be referred to as a “solid state” technique; this process should result in limited damage to the biological. Particle size reduction is accomplished by low temperature grinding (–40º to –50ºC) of solid particles in which shear forces on liquid droplets do not occur. The impact on solid particles transfers energy to the particle that dissipates on fracture and results in only a transient temperature rise. Thus, denaturation, or other destructive processes are limited.

The effectiveness and longevity of pDNA vaccines may be improved by incorporation of pDNA within polymeric delivery vehicles. Administration of naked pDNA leaves the vaccine vulnerable to attack from degradation enzymes that can reduce half-lives to minutes or hours (Kawabata et al., 1995, Luo and Saltzman 2000). Chemical modification of DNA has previously been utilized to protect the vaccine from nucleases and increase vaccine longevity (Benns and Kim 2000, Luo and Saltzman 2000). Modified vaccines have been complexed

with cationic and anionic liposomes, polysaccharides, poly(ethylene glycol), and poly(L-lysine) among others. A drawback to chemical modification has been increases in systemic toxicity resulting from exposure to the complexed chemicals (Luo and Saltzman 2000).

A second alternative involves encapsulation of the plasmid within a polymeric carrier. Biodegradable PLGA systems provide protection for the plasmid, while enabling a sustained and controlled release of the plasmid. Anchordoquy and Koe (2000) reviewed the stability of plasmid-based therapies and suggested that polymeric carrier vehicles such as PLGA may have potential to isolate and entrap DNA. Isolation of the plasmid may prove to be beneficial in reducing negative interactions such as aggregation that leads to loss of biological activity in typical liquid formulations. The application of PLGA to biomedical applications is well established and is based on the biocompatibility of these materials and their degradation products, lactic and glycolic acids (Visscher et al., 1985). Control of plasmid release may improve vaccine efficacy because prolonged availability may enable sustained gene expression (Labhasetwar et al., 1998).

Recent researchers have studied the encapsulation of plasmid-based therapeutics within polymer-based vehicles. Tinsley-Bown et al., (2000) demonstrated the release of a firefly luciferase-derived plasmid from microcapsules of a PLGA. In vitro studies found that the release rate of the plasmid into solution was dependent upon polymer molecular weight. Perez et al., (2001) encapsulated plasmid DNA into nanoparticles of poly(lactic acid) and poly(ethylene glycol) copolymers. In this study, plasmid loadings of 10-12 µg per mg of polymer resulted in a large initial burst of plasmid from the matrix followed by a slower release for 28 days.

Whereas polymeric carriers provide advantages over naked pDNA injections, loss of vaccine effectiveness in terms of physical mass loss and structural rearrangement of pDNA has been observed for encapsulation within polymeric delivery vehicles. Encapsulation efficiency of pDNA within PLGA matrices has varied with technique. Various procedures modified from the traditional double emulsion/solvent evaporation technique have yielded encapsulation efficiencies in the range of 20-50 percent (Tinsley-Brown et al., 2000) and 30–35 percent (Capan et al., 1999). However, Cohen et al., (2000) reported a higher efficiency, 70 percent, for encapsulation of pDNA within nanoparticles of PLGA. In addition to mass loss during the encapsulation procedure, rearrangements of pDNA structure have also been reported. A significant decrease in the percentage of supercoiled pDNA in favor of open circle pDNA has been reported. Tinsley-Brown (2000) reported that 30–40 percent of pDNA was recovered in the supercoiled form with losses being attributed to the open circle conformation. Capan et al., (1999) observed an increased loss of supercoiling, 16 percent, for uncomplexed pDNA. However, through forming of pDNA-poly(L-lysine) complexes, the percentage of pDNA remaining in the supercoiled structure increased to 75–85 percent.

The effectiveness of pDNA vaccines delivered in a PLGA vehicle has been demonstrated in vivo. Cohen et al., (2000) showed that a sustained release

of pDNA from PLGA microparticles increased expression of alkaline phosphatase versus an injection of naked pDNA beyond 7 days. However, injections of polymer-encapsulated pDNA resulted in less expression versus naked pDNA for a period of 72 hours post-injection. This observation was attributed to the reduced availability of encapsulated pDNA with respect to the naked pDNA solution or diminished effectiveness of the vaccine due to rearrangements of pDNA structure. Yet, the polymeric delivery vehicle enabled sustained release of pDNA vaccine. Lunsford et al., (2000) demonstrated persistence of pDNA within specific tissues in mice for a period of 120 days following injection for intramuscularly or subcutaneous injections. Tissues exposed to injections of naked pDNA were observed to be absent of pDNA beyond 15 days post-injection. Vaccine effectiveness may also be benefited by the potential of the polymeric particles to mediate transfection of macrophages during phagocytosis (Cohen et al., 2000).

3. Materials and Methods

3.1. Plasmid Vaccine Production

Preclinical grade plasmid was manufactured and characterized by Puresyn, Inc. (Malvern, PA, Lot No. C25OCT02A). The plasmid under investigation in this study, VR2578, was prepared from a sample procured from the Naval Medical Research Center. The plasmid was 93.9 percent supercoiled with the remainder having an open-circle structure. The size of the VR2578 plasmid was approximately 6000 bp, as measured by gel electrophoresis. Plasmid was stored in 0.9 percent saline at a concentration of 1.77 mg/mL and frozen (-20°C) prior to use.

3.2. Encapsulation of pDNA Vaccine

PLGA microparticles were prepared for use as an adjuvant for the delivery of a plasmid malaria vaccine (VR2578) using a matrix encapsulation technique. A porous biopolymer matrix was impregnated with the plasmid to distribute the vaccine. First, a solution of PLGA in glacial acetic acid (50 mg/mL) was quick-frozen for 10 min. in a dry ice/isopropanol bath. The frozen solution was freeze-dried (Labconco, Model 75040 Freeze Dryer 8, Kansas City, MO) for 48 hours to produce a porous matrix. The void volume of the matrix was approximately 94 percent as determined by foam density. An aqueous solution of VR2578 was loaded into the matrix under reduced pressure such that the percentage of plasmid to PLGA was 1 percent by weight. Finally, the vaccine-loaded foam was quick-frozen in a dry ice/isopropanol bath and freeze-dried for an additional 48 h.

Extrusion of the particles to form rods or cylinders impregnated the plasmid within the polymer matrix. The VR2578/PLGA foams were cryogenically ground (A10 Mill, IKA Works, Wilmington, NC) at -5°C to produce microparticles and then added to a 0.75-in. stainless steel mold. A load of 18,000 lb (corresponding to 40,000 psi) was applied to the mold and rods were extruded through a 1.3-mm die over the course of 1.5 h using a Compac press (MPC 40-1, Juelsminde, Denmark). The temperature of the mold was raised to 50°C in order to plasticize the polymer phase and increase the mobility of the polymer chains to enhance plasmid encapsulation.

Polymer microparticles containing the encapsulated plasmid were produced through a combined grinding/sieving apparatus. The extruded rod was ground on the mill to yield VR2578/PLGA microparticles. Ground particles were sieved through a 43-µm, stainless steel mesh (325 Mesh, Cambridge Wire Cloth, Cambridge, MD) to isolate the smallest-sized particle fractions. Particles were sieved during the grinding process under reduced pressure (Edwards Vacuum Pump, E2M-1, Crawley Sussex, England). In addition, control particles without plasmid were prepared using the same procedure. The yield of VR2578/PLGA collected beyond the sieve was approximately 50 percent of the total mass as measured prior to extrusion.

Microparticle particle size distribution was characterized using light and scanning electron microscopy. Representative samples of VR2578/PLGA particles were placed on a glass slide, viewed under a light microscope (Micromaster I, Westover Scientific, Mill Creek, WA), and photographed (Nikon CoolPix 990 Digital Camera). The size of particles (n=850) was measured from the digitized images using Scion Image for Windows, Release Beta 4.0.2. Particle size mean, median, and distribution were calculated for both particle numbers and mass.

3.3. Characterization of Plasmid Release

The release of VR2578 from the PLGA carriers was characterized in vitro to assess plasmid encapsulation and expected delivery rate from the matrix. PLGA particles containing the encapsulated pDNA with an approximate mass of 10 mg were suspended in 1.5 mL of 0.1 M phosphate buffer saline (PBS). The suspension was incubated at 37°C and shaken at 60 cycles per minute in a PolyScience model 28L water bath. A total of six samples were added to the water bath and the quantity of released plasmid was measured at times of 1, 4, and 24 h and at 7, 21, 28, 35, 42, 49, and 56 days. Upon removal from the water bath, suspended particles were isolated by centrifugation at 50,000 rpm for 10 min. The supernatant solution containing released pDNA was collected with a pipette.

The concentration of pDNA in solution was measured by UV spectroscopy as described by Tinsley-Brown et al. (2000). Approximately 0.5 mL of pDNA solution was added to a quartz cuvette of path length 1 cm and width of 0.2 cm. Solutions of native VR2578 were diluted in PBS to known concentrations to serve as calibration standards. These solutions with known concentrations of pDNA were used to measure the unknown concentrations of pDNA by creating an absorbance versus concentration standard curve. Absorbance was recorded at 260 nm for each solution on a Varian Cary Scan 100 UV/Vis spectrophotometer. A reference absorbance background was provided by a PBS solution that was incubated with control PLGA particles not encapsulated with pDNA.

The quantity of plasmid encapsulated within the polymer particles was measured by accelerating the release of retained plasmid following 56 days of incubation. A basic environment to catalyze polymer degradation and vortex mixing to promote release of the plasmid from the polymer phase resulted in the remainder of encapsulated plasmid to be released. Tinsley-Brown et al. (2000) described this technique for measuring the quantity of plasmid loaded into PLGA

systems. After 56 days of incubation, microparticles and the remaining encapsulated pDNA were isolated from the PBS supernatant. The microparticles were suspended in 1.5 mL of 0.2 M NaOH and incubated at 120°C for 10 min. The basic environment and elevated temperatures promoted degradation of the biopolymer system and release of the pDNA. Following the incubation step, the suspended particles were agitated on a vortex mixer for 1 min. The concentration of VR2578 in solution was measured using UV spectroscopy. For concentration measurement in NaOH, solutions of known pDNA concentrations were created in 0.2 M NaOH for the calibration curve. In addition, the reference background was a NaOH solution incubated with control PLGA particles that did not contain any plasmid.

4. Results

4.1. Microparticle Characterization

Biopolymeric microparticles loaded with the VR2578 plasmid were characterized in this feasibility study to determine potential efficacy of this malaria vaccine. Polymer particle size was characterized using light and scanning electron microscopy. Particles demonstrated a wide particle size distribution ranging from 1 to 45 μm (see Figure 1), but the median particle size was 8 μm based upon particle number and 22 μm by mass (see Table 1). The sieving technique effectively eliminated particles larger than 43 μm and 90 percent of the particles were less than 17 μm in diameter.

Figure 1: Measured Particle Size Distribution

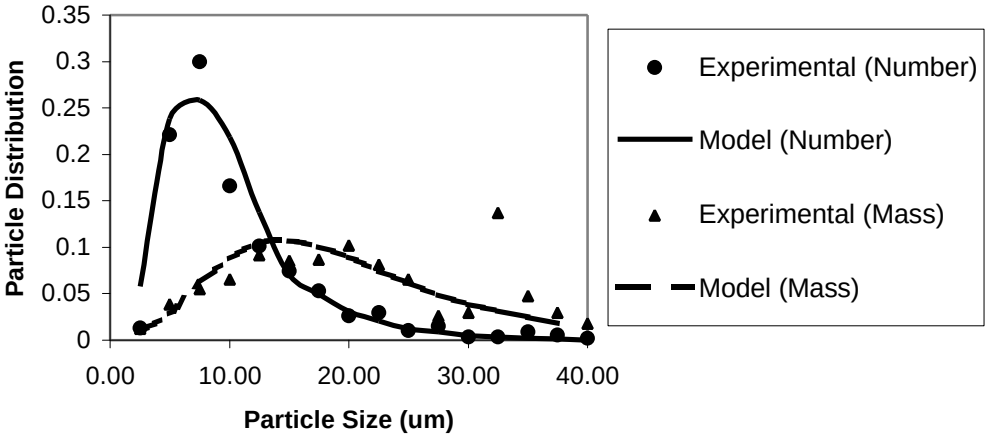


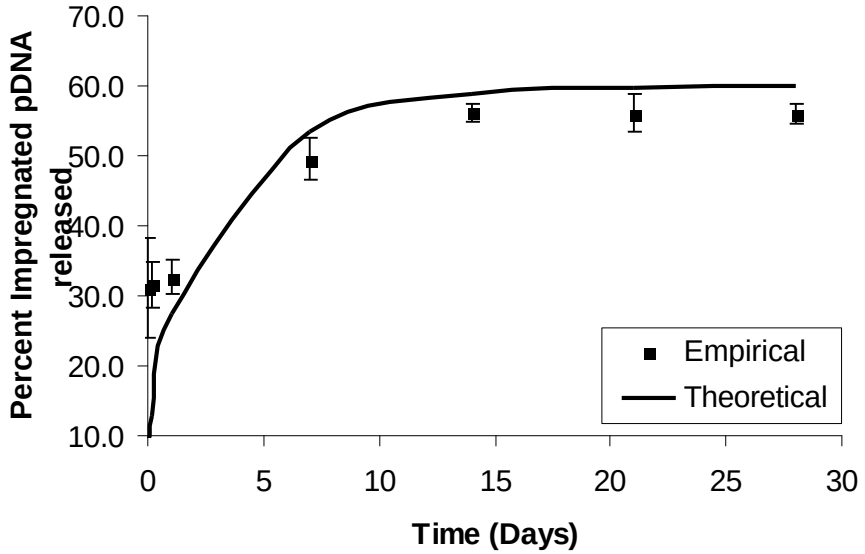
Table 1: Size of Biopolymeric Particles

Property	Based on Number of Particles	Based on Particle Mass
Mean (μm)	9.8	22.9
Standard Deviation (μm)	6.0	9.4
Median (μm)	7.9	21.9

4.2. Characterization of Plasmid Release

An *in vitro* procedure measured the impregnation efficiency and monitored the release profile of VR2578 from the biopolymeric microparticles. Release of the plasmid occurred at a controlled rate for 14 days from microparticles prepared by extrusion (see Figure 2). The plasmid was effectively impregnated using the extrusion technique and the burst effect was significantly reduced. Approximately 30 percent of the plasmid was released immediately upon immersion of the particles into buffer. The remainder of the plasmid was retained within the particles. An additional 30 percent of the total plasmid impregnated within the microparticle system was released through 7 weeks with most of the released VR2578 detected after 14 days. Although the quantity of plasmid released significantly decreased between 14 and 21 days, released VR2578 was detected in the buffer environment through 49 days.

Figure 2: VR2578 Release from PLGA Particles



The release of VR2578 from PLGA microparticles was modeled assuming Fickian diffusion of the plasmid to the buffer environment. For one-dimensional radial release from a sphere of a radius *a*, under perfect sink initial and boundary conditions, with a constant drug diffusion coefficient *D*, Fick's second law may be written as

$$\frac{\partial C}{\partial t} = D\left[\frac{\partial^2 C}{\partial r^2} + \frac{2}{r}\frac{\partial C}{\partial r}\right]$$

where

$t=0 \quad 0<r<a \quad C=C_1$

$t>0 \quad r=a \quad C=C_0$

The solution to Fick's law under the specified conditions is (Crank 1975; Ritger 1987):

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{p^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left[\frac{-Dn^2 p^2 t}{a^2} \right]$$

Using the empirical data collected in this study, the value of *D* calculates as 1.8×10^{-9} m^2/s for early times. The theoretical release profile generally represented the empirical data (see Figure 2). However, the model did not account for the immediate release of plasmid ("burst effect") from the microparticle system.

5. Discussion

This Milestone 1 feasibility study demonstrated potential efficacy of this biopolymeric system for application to the delivery of a plasmid malaria vaccine. The feasibility study focused on the characterization of particle size distribution and the impregnation of the plasmid within the biopolymer. Particle size was optimized for this matrix system given that small particles promote uptake of polymer particles by antigen presenting cells. Impregnation of the plasmid within the polymer carrier controlled the "burst effect" associated with plasmid release following administration.

The combination cryogenic grinding and sieving procedures developed during Milestone 1 effectively reduced the particle size of the biopolymeric vaccine system. Particle sizes on the order of 10 μm and less were desired for particle uptake by antigen presenting cells. Microscopic evaluation of particles determined that 90 percent of the particles were less than 20 μm and the median diameter was 8 μm. The smallest particle fractions passed through a 43-μm sieve during the grinding procedure. Sieves smaller than 43-μm were not permissive as the particles aggregated on the mesh, and consequently, the yield of particles within the desired size range was limited. Therefore, the particle production technique was optimized based upon final particle size and yield of particles, which was at least 50 percent by mass of the starting materials.

An extrusion technique promoted incorporation of the plasmid vaccine within the microparticle system and controlled the "burst effect" *in vitro*. Particles prepared via compression of the DNA loaded polymer matrix at 40,000 psi for 60 min. as conducted during Phase I studies exhibited an "initial burst" of approximately 60-90 percent of the total incorporated DNA. In this study, PLGA matrices were extruded through a 1.3-mm die at 40,000 psi and 50°C. The pressure and temperature used in this part of the effort were selected such that a DNA plasmid could be incorporated with the PLGA while the polymer was in an amorphous state, without the likelihood of denaturing the plasmid. Following extrusion of the polymer, PLGA returned to a glassy state to form a rod of polymer. Particles were generated by grinding this plasmid/polymer rod and sieving to retain the smallest particle fraction.

Impregnation of the plasmid was characterized by immersing particles into a buffer to simulate physiological conditions. Approximately 30 percent of the incorporation plasmid was immediately released from the system. This "burst effect" was significantly more controlled when compared to the Phase I results. Furthermore, an additional 30 percent of the plasmid was released at a controlled

rate up to 14 days *in vitro*. Plasmid aggregation within the particles and/or absorption of the plasmid onto PLGA may have prevented the remainder of the plasmid from being released. Overall, the release profile of the plasmid was comparable to other therapeutics incorporated within PLGA systems. Release of the plasmid was modeled using a diffusion model in order to plan and optimize effective doses of VR2578/PLGA particles for future preclinical studies.

6. Conclusions

The plasmid vaccine incorporated within a PLGA matrix system demonstrated potential efficacy in this study for application for use against malaria. During Phase I studies, mice were inoculated with a VR2516/PLGA vaccine via subcutaneous administration. This route of administration was not effective for provoking an immune response. In order to further develop the biopolymeric vaccine system, particle size and plasmid incorporation were optimized in Milestone 1 of this Phase II project. Reduction in particle size of the biopolymeric carrier was sufficient for intramuscular administration of the VR2578/PLGA system in a mouse model, a more reasonable simulation of the anticipated clinical treatment. Furthermore, the achieved particle size indicated that this biopolymeric system has the potential to promote particle uptake by antigen presenting cells *in vivo*. Improvements in the particle production technique controlled the "burst effect" and effectively incorporated the plasmid within the polymer particles. Characterization of the plasmid release profile was modeled in order to estimate effective doses of the VR2578/PLGA system when the vaccine is applied to preclinical models as proposed during Milestone 2 of this Phase II project.

7. References

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